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Vanadium-Containing Blood Cells (Vanadocytes) Show No Fluorescence Due to the Tunichrome in the Ascidian, *Ascidia sydneiensis samea*

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ABSTRACT—Ascidians belonging to the family Ascidiidae are known to accumulate vanadium ions from seawater to levels in excess of one million times the level in seawater and to maintain the vanadium ions in a reduced form. A tunichrome which appears to be involved in the accumulation and reduction of vanadium ions produces an autonomous fluorescence upon excitation with blue-violet light. Among six different types of ascidian blood cell, the morula cell, which emits fluorescence brightly, has been thought to be the vanadium-containing blood cell (vanadocyte) and, consequently, it has been suggested that the intensity of fluorescence is indicative of the concentration of vanadium ions in the blood cell.

In the present experiments, after ascidian blood cells were fractioned into the various subpopulations by means of Ficoll density gradient centrifugation, the level of vanadium in each subpopulation was determined to ascertain which type of blood cell is the true vanadocyte in *Ascidia sydneiensis samea*. The autonomous fluorescence from the vanadocyte was also monitored with a fluorescence microscope. Consequently, we found that the subpopulation of morula cells that fluoresced brightly did not contain vanadium, whereas the subpopulation of signet ring cells, which did not emit fluorescence, contained high levels of vanadium.

INTRODUCTION

The ability of ascidians to concentrate vanadium ions, to levels in excess of one million times the level in seawater, is a source of special fascination [1, 2]. A tunichrome, which can be extracted from the blood cells of *Ascidia nigra,* has been proposed to be involved in the accumulation of vanadium ions from seawater [1, 3–5]. This substance has been reported to emit a specific autonomous fluorescence upon excitation with blue-violet light [4, 6]. Among several different types of ascidian blood cell examined, the strongest fluorescence that could be ascribed to the tunichrome was observed in the morula cell. Thus, it was suggested that the intensity of fluorescence is indicative of the concentration of vanadium ions in the cells [4].

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However, we have already verified that the morula cell contains no vanadium, whereas the signet ring cell contains a very high level of vanadium ions, in the case of *A. ahodori.* We based our conclusions on the results of cell fractionation techniques, neutron activation analysis and electron spin resonance spectrometry (ESR) [7].

In the present experiments, we examined whether the signet ring cell (vanadocyte), separated by Ficoll density gradient centrifugation, emits an autonomous fluorescence due to the tunichrome, in order to verify any participation by this substance in the accumulation of vanadium ions in ascidian blood cells from seawater.

MATERIALS AND METHODS

Ascidia sydneiensis samea were collected in the bay of Nanao, Ishikawa Prefecture, Japan, and were maintained in an aerated seawater aquarium at 18° C. Blood, drawn by making an incision through the lower part of the tunic and puncturing

the mantle, was suspended in artificial seawater (ASW) that contained 460 mM NaCl, 9 mM KCl, 33 mM Na_2SO_4, 6 mM $NaHCO_3$, 1 mM EDTA(ethylenediamine-tetraacetic acid) and 5 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (pH 7.0) to avoid clotting. The suspension was separated into blood cells and plasma by centrifugation at $300\times g$ for 10 min at 10°C. The pellet obtained was resuspended at a concentration of about 10^7 cells/ml in ASW and is referred to as "washed cells". Thereafter, cell fractionation by Ficoll density gradient centrifugation was carried out in a manner similar to that described previously [7]. Ficoll type 400 (Pharmacia Fine Chemicals) was dissolved in ASW to final concentrations of 34.0, 18.0, 14.5 and 4.0% (w/v) and discontinuous gradients were prepared in 10-ml centrifuge tubes. One ml of washed cells was layered onto each gradient and tubes were centrifuged at $300\times g$ for 20 min at 10°C. Layers of cells were gently pipeted from the top of the tubes. Each layer of cells obtained in this way was washed twice with ASW by centrifugation at $350\times g$ for 10 min in order to remove Ficoll 400 and was resuspended in a small amount of ASW. The fractioned populations of cells were subsequently used for determination of levels of vanadium by neutron activation analysis at the Institute for Atomic Energy of Rikkyo University, Yokosuka, Japan [2]. The blood cells which were resuspended in a small amount of ASW were observed with a standard bright field microscope and a fluorescence microscope (Nikon). They were also observed after vital staining with neutral red, nile blue and Janus green.

RESULTS

Morphology and fluorescence of blood cells

We were able to recognize six different types of cell: the giant cell, signet ring cell, morula cell, compartment cell, pigment cell and hyaline leucocyte. These types of cell were classified mainly according to the criteria of Wright [8] and Rowley [9].

Subpopulations of the blood cells are present at variable proportions in individuals in this species. The giant cell was the second most abundant cell type accounting for 21 to 30% of the total cells. This cell was very large and spherical or irregularly shaped. It was 40 to 80 μm in diameter and contained a single, very large, fluid-filled vacuole which occupied most of the cell (Fig. 1A). The vacuole was coloured faint red and faint violet after staining with neutral red and nile blue, respectively. The cell weakly emitted a pale green fluorescence (Fig. 1a). The giant cell is probably analogous to the nephrocyte [8]. It seems however reasonable that the cell should be designated as a giant cell because it is not apparent that the giant cell is involved in excretion.

The morula cell was round to ovoid, 8 to 10 μm in diameter. As shown in Figure 1B, the morula cell in this species exhibited refractive cytoplasm under bright field illumination and a few cells appeared typical berry-like shape, differing from the morula cell in the other species. It was accounted for 6 to 12% of the total population. This cell appeared red, blue-green and green after staining with neutral red, nile blue and Janus green, respectively, and emitted autonomous fluorescence with a yellow green colour upon excitation with blue-violet light (Fig. 1b).

The signet ring cell, 10 to 12 μm in diameter, which comprised about 32 to 44% of the total population of cells, predominated. This cell was characterized by a single and fluid-filled vacuole which displaced the nucleus and cytoplasm to the periphery of the cell (Fig. 1C). A single, small, refractive vesicle was suspended in the vacuole. Such vacuole was coloured a faint red after staining with neutral red. The small vesicle was dyed a red and green with neutral red and Janus green, respectively. No fluorescence was detected from this cell upon excitation with blue-violet light (Fig. 1c).

The compartment cell, was ovoid, 6 μm in diameter, and accounted for 15 to 21% of the total population of cells (Fig. 1D). This cell was dyed a red with neutral red. The granules in the cytoplasm appeared deep red and green after staining with neutral red and Janus green, respectively. No fluorescence was detected from the compartment cell (Fig. 1d).

Pigment cells (Fig. 1E) were relatively rare. The

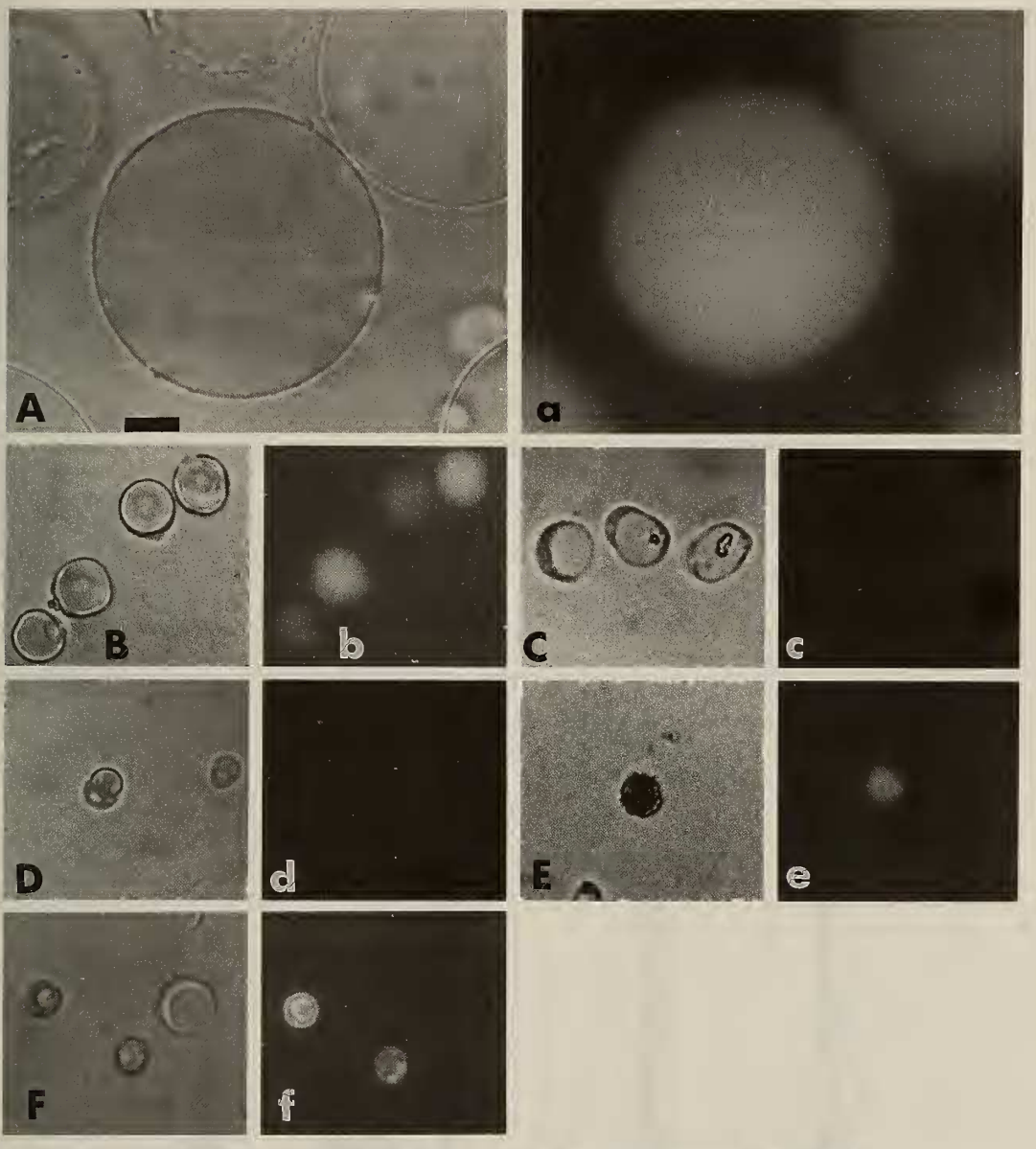

FIG. 1. Blood cells of *Ascidia sydneiensis samea* were observed with a bright field microscope (A-F) and a fluorescence microscope (a-f). The giant cell, the second most abundant cell type, was 40 to 80 μm in diameter and contained a single, very large, fluid-filled vacuole (A). This cell weakly emitted a pale green fluorescence upon excitation with blue-violet light (a). The morula cell had refractive cytoplasm (B) and a few cells appeared typical berry-like shape. This cell emitted autonomous fluorescence with a yellow green colour (b). The signet ring cell, which contained large amounts of vanadium, was characterized by a single and fluid-filled vacuole and refractive vesicle in the vacuole (C). This cell type was predominant in the blood cells. From this cell no fluorescence was detected upon excitation with blue-violet light (c). The compartment cell was small and ovoid, accounting for 15 to 21% of the total population of cells (D). No fluorescence was also detected from this cell (d). The pigment cell shows red, dark orange or brown colour (E) and emitted bright coloured fluorescence (e). The hyaline leucocyte (F) fluoresced most brightly among the blood cells in this species (f). Scale bar indicates 10 μm.

pigment cell emitted bright orange coloured fluorescence (Fig. 1e). The cells shown in Figure 1F seem to be hyaline leucocytes and fluoresced most brightly among the blood cells in this species (Fig. 1f).

Cell fractionation

The blood cells were partitioned into four discrete layers that contained various subpopulations, after Ficoll density gradient centrifugation. Most

TABLE 1. Distribution of each subpopulation of blood cells prior to and following separation by centrifugation on a Ficoll density gradient

	Total cell number	Giant cells	Signet ring cells	Morula cells	Compartment cells
Washed cells	79,422±16,250 (100.0)	20,545±10,789 (63.9)	29,398±7,274 (14.0)	8,344±1,210 (3.2)	13,936±2,370 (8.5)
Layer 1	2,294±808 (100.0)	1,466±555 (63.9)	321±179 (14.0)	73±66 (3.2)	194±59 (8.5)
Layer 2	3,096±1,189 (100.0)	—	2,816±1,119 (91.0)	—	217±70 (7.0)
Layer 3	875±164 (100.0)	—	465±171 (53.1)	—	396±56 (45.3)
Layer 4	517±167 (100.0)	—	—	471±159 (91.1)	—
Total number of cells recovered	6,782	1,466	3,602	544	807

Each number of blood cells is shown in 1,000 cells and as the mean±standard error. Figures in the parenthesis express % of total number of blood cells in the washed cells and each layer.

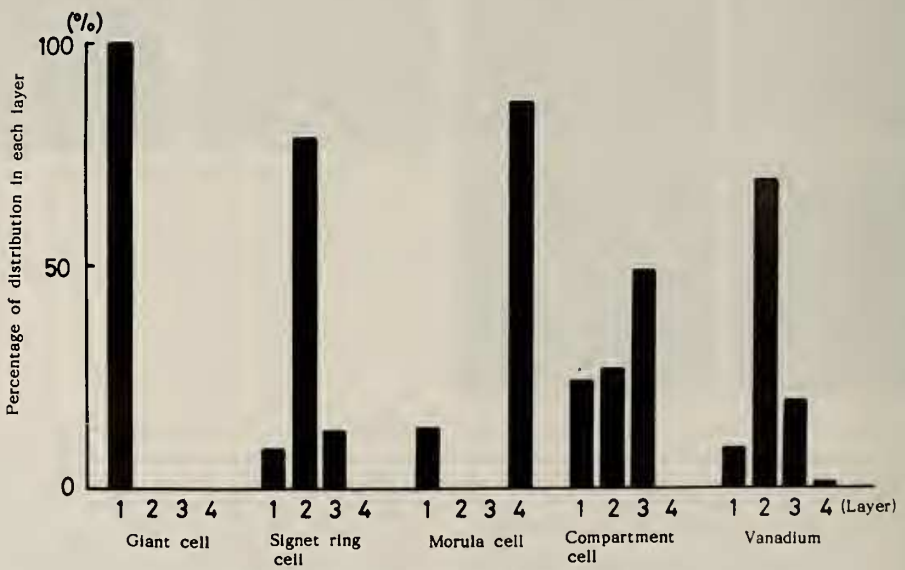

FIG. 2. Comparison of the patterns of distribution of giant cells, signet ring cells, morula cells and compartment cells with that of vanadium ions after density gradient centrifugation of blood cells of *A. sydneiensis samea*. Histograms depict the relative numbers of each type of cell and the amounts of vanadium distributed in the four layers of cells as percentages of the total number of cells and the total amount of vanadium. Each bar represents the average of results of five trials. The pattern of distribution of vanadium is similar to that of the signet ring cells but is different from those of the other cell types.

of the separated blood cells in each layer did not take up eosin Y, indicating that they were still alive. The results obtained represent the average of results of five trials±S.E. (standard error) and are shown in Table 1. About 6 to 12% of each subpopulation were recovered from the various layers, which low rates were due to sacrifice the recovery rate to obtain purer subpopulation of blood cells.

The percentages of giant cells, signet ring cells, morula cells and compartment cells distributed among each of four layers, are presented graphically in Figure 2. All of the giant cells were gathered in layer 1, suggesting that they were of low density. About 79% of the signet ring cells were present in layer 2 and the remaining 8% and 13% were found in layer 1 and 3, respectively. Almost all of the morula cells (88%) were present in layer 4, indicating that this cell type has highest density among the blood cells in this species. Half of the subpopulation of the compartment cells was found in layer 3 and the other half was divided between layer 1 and 2.

Vanadium content

Neutron activation analysis revealed that 127.5 μg of vanadium was contained in the washed blood cells and 14.7 μg of the metal, which corresponded to about 12% of the initial amount in the washed cells, was recovered from the fractioned blood cells as shown in Table. 2. The highest percentage of vanadium distributed among four layers was found in layer 2, in which about 70% of the vanadium was present. The remaining 9 and 20% of vanadium were distributed in layer 1 and 3, respectively.

TABLE 2. Vanadium content of layers separated by Ficoll density gradient centrifugation

	Total content (ng)
Washed cells	127,540±32,240
Layer 1	1,370±640 (9.3%)
Layer 2	10,220±3,640 (69.4%)
Layer 3	2,900±820 (19.7%)
Layer 4	230±150 (1.6%)
Recovered vanadium after separation	14,720

Each value represents the average of five trials± standard error. Figures in the parenthesis express % of recovered vanadium.

The pattern of distribution of vanadium was compared with those of blood cells in Figure 2. This pattern was very similar to that of the signet ring cells, but was clearly different from that of the morula cells and of the other cell types. Furthermore, the following data strengthen evidence that the signet ring cell is the vanadocyte. As has been pointed out, the proportion of each type of blood cell varied in individuals in this species. Hence, the signet ring cells recovered from layer 2 also varied in number through 1,664,000 to 4,867,000 cells and vanadium content in layer 2 rose and fell in proportion to the cell number. When the vanadium content per 1000 signet ring cells in layer 2 was calculated in each trial, the values were of a very narrow range of 3.04 to 4.11 ng/1000 cells (average value±S.E. was 3.71±0.4 ng/1000 cells). Such tight correlation of vanadium content with the cell number could not be found out in the other type of blood cell.

DISCUSSION

The vanadium ion dissolved in seawater is in the +V oxidation state at concentrations of about 35 nM [10, 11]. Some ascidians concentrate these ions 10^6-fold in their blood cells and store the metal ion in its reduced +III and/or +IV states [7, 12–16]. Macara *et al.* [1] isolated a tunichrome from the ascidian bllod cells, which serves as a good agent for forming complexes with vanadium ions and which reduces the metal ion to its reduced form. The tunichrome emits fluorescence when it is excited with blue-violet light and is present with vanadium at appoximately equimolar concentrations [1]. The concentration of vanadium has, therefore, been estimated from the intensity of fluorescence of the tunichrome in each type of blood cell. In consequence, concentrations of both vanadium and tunichrome were thought to be in the order, morula cell>compartment cell › signet ring cell [4].

Although the morula cell unequivocally emitted fluorescence in this experiment (Fig. 1), the combind results of cell fractionation and neutron activation analysis have revealed that the morula cell dose not contain vanadium, while the signet ring cell which dose not fluoresce contains large amounts of vanadium (Tables 1 and 2). In fact, the pattern of distribution of vanadium among the fractioned layers corresponded clearly to that of the signet ring cells as shown in Figure 2. Moreover, the vanadium content per 1000 signet ring cells in layer 2 was almost consistent in each trial. Based on these results, it could be concluded that the actual vanadocyte involved in the accumulation of vanadium must be the signet ring cell in *A. sydneiensis samea.*

If the tunichrome is involved in the reduction and accumulation of vanadium ions in the ascidian blood cells, it would be necessary that the tunichrome should be contained in the signet ring cell, which is the vanadocyte. However, no fluorescence due to the tunichrome was detected in this cell (Fig. 1). In a different species of *A. ahodori,* a similar finding that the signet ring cell did not emit fluorescence was obtained from preliminary experiments in which the blood cells were not fractioned [17]. Tunichrome B-1, one of the tunichromes, has been isolated from non-separated blood cells of *A. nigra* and its chemical structure has been determined. It consists of three units of hydroxy-DOPA (3, 4-dehydroxyphenylalanine) [18], and it must have the ability to reduce an oxide to its reduced state, as shown in the report by Macara *et al.* [1]. There is, however, no evidence that this substance is involved in the reduction and accumulation of vanadium ions in the vanadocytes in ascidian blood.

The fluorescence emitted by ascidian blood cells [1, 3, 4, 6] cannot be attributed solely to the tunichrome. It is well known that there are several kinds of autonomous fluorescent substance, for example, lipids, vitamins and porphyrins, in living cells. Therefore, the finding of fluorescence does not always provide evidence for the presence of a tunichrome.

We have extracted a vanadium-binding substance which we have called vanadobin from the blood cells of *A. sydneiensis samea.* This substance is colourless and can maintain the vanadium ion in the vanadyl form (VO(IV)), even under aerobic conditions. Moreover, this substance has an affinity for exogenous vanadium ions (V) and contains a reducing sugar [16]. Taking all the above data into account, we suggest that it is not the tunichrome but rather the vanadobin that is the substance involved in the accumulation of vanadium ions from seawater in ascidian blood cells.

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